REMARKS

Claims 1-3, 7-18, 40 and 42-49 remain in this application. Claims 4-6, 19-39 and 41 have previously been canceled without prejudice. Claims 1, 3, 9, 14-15, 40, 42, 44 and 48 currently being amended.

Claim 1 has been amended to specify that the basal medium in step b) is a "first basal medium", and the medium in step c) is a "second basal medium". The terms "first" and "second" have been inserted for the purpose of clarity. Support for this amendment can be found throughout the specification, for example at page 21, bottom paragraph (lines 23-27); page 24, middle paragraph (lines 11-21); page 26, top paragraph (lines 4-11); page 27-28; page 28, paragraph spanning pages 28-29 (page 28, line 24 to page 29, line 2); page 29, bottom paragraph (lines 26-29); Example 5; and Example 6.

Claim 3 has been amended to remove the term "or seedlings", and claim 11 has been amended to specify that the explant is a tissue selected from the group consisting of a seed, petiole, hypocotyl, stem, cotyledon and leaf. Support for the latter amendment can be found throughout the specification, for example page 24, lines 1-2; page 25, middle paragraph (lines14-16); page 27, middle paragraph (lines 13-15); page 28, middle paragraph (lines 16-18); and page 29, last line to page 30, lines 2.

Claim 9 has been amended to correct a typographical error. Support for this amendment may be found in claim 9 as originally filed. Claim 9 has also been amended to show "-1" in a superscript position.

Claims 14 and 15 have been amended to show "-1" in a superscript position.

Claims 40 and 42 have been amended to depend from claim 1, rather than claim 4. Claim 42 has also been amended to show "-1" in a superscript position.

Claim 44 has been amended to correct a spelling error.

Claim 48 has been amended to correct a clerical error.

For the reasons given below, Applicants submit that the amended claims are in condition for allowance and notification to that effect is earnestly solicited.

Claim Rejections Under 35 U.S.C. § 112

Claim 1 is rejected under 35 U.S.C. 112, second paragraph as being indefinite, as Examiner alleges that it is unclear whether the basal medium from step b) is the same as the basal medium from step c). Examiner further contends that this term is not defined in the specification or claims. Applicant respectfully disagrees with Examiner's objection.

Applicant has amended claim 1 to specify that the basal medium in step b) is a "first basal medium", and the medium in step c) is a "second basal medium". Applicant submits that it would be clear to a person of skill in the art, reading the present specification (e.g., page 21, bottom paragraph (lines 23-27); page 24, middle paragraph (lines 11-21); page 26, top paragraph (lines 4-11); page 27-28; page 28, paragraph spanning pages 28-29 (line 24 to page 29, line 2); page 29, bottom paragraph (lines 26-29); Example 5; and Example 6) that the explant with regenerated tissue can first be cultured on basal medium, then transferred to a fresh, or second basal medium supplemented with an additive of interest (nutrient mineral element) for subculture. Therefore, Applicant submits that the clarifying amendment finds support in the specification.

Furthermore, Applicant submits that the term "basal medium" is defined in the specification on page 21, line 19 to page 22, end of Table 1. Table 1 of the specification provides several examples of basal media that are well known in the art, for example Murashige & Skoog, and Gambourg media. The use of these and similar media (e.g. Linsmaier & Skoog) in the cited prior art (e.g. Stojakowska, Cellarova et al., and Dodds) supports the fact that a person of skill in the art would be familiar with these media. Furthermore, these types of culture media are readily recognized within the prior art as media for plant cell culture, see for example, Potrykus and Shillito (1988), *in* Methods for Plant Molecular Biology, Weissbach and Wessbach (eds), Academic Press, San Diego, pp. 370-373; copy enclosed as Exhibit A).

Therefore, Applicant maintains that a person of skill in the art would understand the meaning of "basal medium", upon reading the present description, and use of their common general knowledge.

Claim 3 is rejected under 35 U.S.C. 112, second paragraph due to an antecedent problem with respect to the seedlings. In response, Applicant has amended claim 3 to remove the expression "or seedlings".

Examiner rejected claim 11 under 35 U.S.C. 112, second paragraph, stating that it is unclear how an explant could be selected from the seed, and that there is no antecedent basis for the seed, petiole, hypocotyls, stem, cotyledon, and leaf. Claim 11 has been amended to specify that the explant is a tissue selected from the group consisting of a seed, petiole, hypocotyl, stem, cotyledon and leaf. Applicant submits that the use of the indefinite article "a" addresses the antecedent problem noted by Examiner. Applicant also maintains that the specification clearly indicates that any such tissues are suitable for use in micropropagation (page 24, lines 1-2; page 25, middle paragraph (lines14-16); page 27, middle paragraph (lines 13-15); page 28, middle paragraph (lines 16-18); and page 29, last line to page 30, line 2). Furthermore, Applicant points out to Examiner that the use of a seed as an explant is known within the art (see, for example, Smith (1988), *in* Methods for Plant Molecular Biology, Weissbach and Wessbach (eds), Academic Press, San Diego, pp. 343-346, copy enclosed as Exhibit B).

Examiner rejected claims 40 and 42 under 35 U.S.C. 112, second paragraph, stating that they depend from a cancelled claim. In response, Applicant has amended the claims to depend from claim 1, rather than claim 4.

Claim 45 is rejected under 35 U.S.C. 112, second paragraph because the medium contains one or more plant growth regulator, while claim 44 (from which claim 45 depends) specifies a basal medium lacking a plant growth regulator. Applicant respectfully traverses Examiner's objection. Applicant points out to Examiner that claim 45 claims that in the step of culturing (i.e., step a)) of claim 44, the induction medium comprises one, or more than one plant growth regulator. It is submitted that the induction medium of step a) and the basal medium of step b) of claim 44 are two distinct media used in two distinct steps of the claimed method. Therefore, it is maintained that it would be clear to a person of skill in the art that the absence of plant growth regulators in the basal medium does not preclude the presence of plant growth regulators in the induction medium.

Removal of the rejection to claims 1, 3, 11, 40, 42 and 45, and their associated dependant claims under 35 U.S.C. 112, second paragraph is respectfully requested.

Claim Rejections Under 35 U.S.C. § 102/103

Claim 47 is rejected under 35 U.S.C. Section 102(b) as allegedly being anticipated by, or in the alternative under 35 U.S.C. Section 103(a) as obvious over Cellarova et. al. Examiner contends that Cellarova et. al. grows plants in the presence of Ca and Zn, and that the levels of these additives would be elevated when compared to plants grown on a basal medium.

Claim 47 is directed to a phytopharmaceutical plant prepared by the method of claim 1. Claim 47 further defines that the plant of claim 1 comprises an elevated level of the additive of interest "compared to a plant grown in said basal medium in the absence of said additive of interest". Claim 1 specifies that the basal medium is supplemented with from about 50 to about 200 mg/L of an additive of interest selected from the group consisting of a vitamin, boron, chromium, cobalt, copper, iron, lithium, iodine, manganese, molybdenum, nickel, selenium, silicon, tin, vanadium and zinc. Applicant notes that these elements are either micronutrients or a vitamin and are not present in basal media at the amounts indicated. Furthermore, calcium is a macronutrient and not a micronutrient.

Cellarova, (last sentence of page 267) uses a basal medium containing macroelements and microelements according to Linsmaier and Skoog. The composition of the Linsmaier and Skoog (LS) media, along with the composition of other basal media commonly used within the art, was provided in the response to the previous office action. Applicant agrees with Examiner that the LS medium contains calcium and zinc. However, calcium is a macronutrient and is not listed as an additive in claim 1, and zinc is only present as a micronutrient at an amount of 8.6 mg/L.

Cellarova et. al. do not teach or suggest the supplementation of the LS medium with an additive of interest, for example a microelement *in addition to those already present in the medium*. Therefore, Cellarova et. al. do not teach or suggest the supplementation of the LS medium with from about 50 to about 200 mg/L of zinc and growing a plant on this supplemented basal medium.

Applicant submits that Examiner's comparison of a plant grown in a medium containing calcium and zinc to a plant grown on a basal medium lacking these elements is incorrect. Applicant maintains that, in accordance with claim 47, the amount of additive of interest in a plant grown in basal medium that has been supplemented with about 50 to about 200 mg/L of the

additive of interest, is to be compared to the amount of additive of interest in a plant grown in *the same basal medium that has not been supplemented.* For example, if the additive of interest were zinc, and the basal medium were LS medium, then the amount of zinc in a plant grown in a media containing LS, supplemented with 50-200 mg/L zinc, would be compared to the amount of zinc in a plant grown in LS. While the plant grown in LS alone would comprise a small amount of zinc, the plant grown in LS plus 50-200 mg/L zinc would comprise a significantly greater amount of Zn. This can be readily seen with reference to Figure 7, and supporting text page 49, middle paragraph (Example 6) of the present application, where plants are grown in basal media ("0" mg/L zinc added to the basal media), and media supplemented by an additional 50 to 200 mg/L zinc. The amount of zinc in plants cultured is shown. A clear 10-fold increase in the levels of zinc can be seen within the plant tissues when the basal media is supplemented with 50 mg/.L zinc, when compared to a plant grown in the basal media alone.

With respect to Examiner's comment that in the absence of any definition of "basal", water could be considered a basal medium, Applicant submits that to one of skill in the art, upon reading the specification would readily understand the term "basal media". As discussed above, Table 1 of the specification provides several examples of basal media that are well known in the art, for example Murashige & Skoog, and Gambourg media. The use of these and similar media (e.g. Linsmaier & Skoog) in the cited prior art (e.g. Stojakowska, Cellarova et al., and Dodds) supports the fact that a person of skill in the art would be familiar with these types of media. These and other culture media are repeatedly identified in the prior art as media suitable for plant cell culture (e.g. Smith (1988), pp 346-7; and Potrykus and Shillito (1988), pp. 367-373, *in* Methods for Plant Molecular Biology, Weissbach and Wessbach (eds), Academic Press, San Diego; copies enclosed as Exhibits B and A, respectively). Furthermore, Applicant submits that providing that a plant demonstrates an increase in the amount of an additive of interest as defined in claim 47 when compared to a plant grown in the absence of the additive of interest, where the additive of interest is as defined within claim 1 (i.e. the additive of interest is provided at an amount from about 50 to about 200 mg/L, and it is selected from the group consisting of a vitamin, boron, chromium, cobalt, copper, iron, lithium, iodine, manganese, molybdenum, nickel, selenium, silicon, tin, vanadium and zinc) then any media, as would be known to one of skill in the art, may be used as a "basal media".

There is no teaching, or suggestion in Cellarova et. al. that the basal media is to be supplemented with about 50 to about 200 mg/L, of a compound selected from a vitamin, boron, chromium, cobalt, copper, iron, lithium, iodine, manganese, molybdenum, nickel, selenium, silicon, tin, vanadium and zinc. Therefore, Applicant submits the subject matter of claim 47 is not disclosed or suggested by Cellarova et. al., and removal of the rejection under 35 U.S.C. 102(b), and/or 35 U.S.C. Section 103(a) is requested.

SUMMARY

It is respectfully submitted that the above-identified application is now in a condition for allowance and favorable reconsideration and prompt allowance of these claims are respectfully requested. Should the Examiner believe that anything further is desirable in order to place the application in better condition for allowance, the Examiner is invited to contact Applicants' undersigned attorney at the telephone number listed below.

Respectfully submitted,

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A. Genome Structure

[23] Protoplasts: Isolation, Culture, Plant Regeneration

By INGO POTRYKUS and RAYMOND D. SHILLITO

Protoplasts are isolated, single, and "naked" plant cells. Some have the potential to (1) regenerate a cell wall, (2) dedifferentiate, (3) divide mitotically and proliferate to form unlimited growing cell clones, and (4) differentiate shoot and root meristems (or embryos) which grow out to regenerate complete plants. Protoplasts are thus potentially totipotent individuals at the single cell level. Their freely accessible plasma membrane makes them, in addition, ideal experimental systems for many kinds of genetic manipulation.

The following article is organized in three parts:

1. An idealized example of protoplast isolation will show the steps involved in protoplast isolation and culture.

2. A general section will discuss the parameters involved in the developmental sequence from protoplast to plant.

3. An experimental section will give a collection of detailed laboratory protocols for protoplast isolation, clonal proliferation, and plant regeneration which are in routine use in independent laboratories. It is recommended that one should gain expertise with one of these easy protocols before approaching a new experimental system. It is not absolutely necessary, but it might be helpful to visit an established protoplast laboratory.

An Idealized Example

An idealized example will be given first to give a general outline of the method of protoplast isolation. For specifics the reader is referred to the detailed protocols.

Plants of a competent genotype of a herbaceous dicot (*Petunia hybrida*: see also protocol 2 below) are grown in potting compost in clay pots in a controlled environment (12 hr light, 5000 lux, 27°/20° day/night temperature, 50/70% relative humidity) and watered with a 0.1% commercial fertilizer solution at 8.00 am and 4.00 p.m.

Leaves expanded to two-thirds their final size are harvested from plants at the 7–9 leaf stage, washed with tap water, and surface sterilized by brief immersion in 70% ethanol followed by 10 min incubation in 0.01% w/v mercuric chloride containing a wetting agent. This is carefully washed off by five rinses each of 5 min with sterile distilled water. The leaves are then carefully arranged in stacks of 6, wet with osmoticum; and carefully

cut into fine cross sections. These are collected and transferred to a small volume of enzyme solution and vacuum infiltrated to replace the intercellular air with enzyme solution. Samples of ~500 mg tissue are incubated in 10 ml of enzyme solution in 9-cm petri dishes at 28° for ~3 hr. The process of protoplasting is observed from time to time under the inverted microscope. Gentle shaking helps to release protoplasts from the digested tissue toward the end of the incubation period.

The total digest is filtered through a 100-μm stainless-steel sieve, mixed with an equal volume of osmoticum, and distributed into capped centrifuge tubes. These are centrifuged (5 min, 60 g) to sediment the protoplasts without sticking them too tightly to the bottom of the tube. The supernatant is carefully pipetted off, the sediment resuspended in the residual 0.5 ml of liquid, and the tube filled with fresh osmoticum. After 2 repetitions of this process, the protoplasts (Fig. 1) are taken up in culture medium, a sample taken for counting, and the population density adjusted to 2×10^4/ml. Aliquots are pipetted into petri dishes to give a thin liquid layer which just covers the bottom. The dishes are sealed with Parafilm and incubated at a constant temperature of 26° in the dark.

Cell wall regeneration and dedifferentiation are visible after one day (Fig. 2) and the first divisions after three (Fig. 3). The division frequency can be estimated 7–10 days after isolation by counting dividing and nondividing protoplasts in representative fields and calculating the percentage of the total surviving population which has divided at least once. At this time the suspension is diluted with 1/3 volume of culture medium with reduced osmotic pressure and growth regulator concentration. This is repeated weekly and after a total of 4 weeks protoplast-derived colonies become visible to the naked eye (Figs. 4 and 5). When these have reached a size of 1–2 mm in diameter (Fig. 5), the plating efficiency (colony-forming efficiency) is established by calculating how many proliferating cell clones have developed per 100 protoplasts originally plated.

At this stage cell colonies are transferred onto the surface of agar-solidified medium for further proliferation at low osmotic pressure. After a total of 2 months the colonies are large enough (>5 mm in diameter, Fig. 6) to be transferred to regeneration media which promote the development of meristems and the outgrowth of shoots (Fig. 7) and roots (Fig. 8). After a further month a fraction of the clones will have regenerated multiple shoots (percentage regeneration efficiency) which are rooted as cuttings on a medium with a low auxin concentration (Fig. 9). Two weeks later the rooted shoots are carefully washed free of agar, potted into a fine potting compost, and adjusted slowly to a low relative humidity. The plants can then grow further without any special care (Fig. 10).

Parameters Affecting Protoplast Isolation and Proliferation and Plant Regeneration

The developmental sequence from protoplast to plant (Figs. 1–10) depends upon numerous parameters and can fail at many points. A discussion of these factors will help in understanding the complexity of the phenomenon, and may serve as a guideline to identify mistakes in experiments which fail to reproduce published protocols or where new systems are under study.

Competence

The key factor which decides whether or not a protoplast will divide, proliferate, and regenerate into a plant is a phenomenon which we can observe, but about which we have little solid information: competence. This term is used to describe the ability of a cell to respond in specific ways to specific stimuli during development.[1] The competence on which the developmental route from protoplast to plant depends describes the capacity of specific plant cells to respond to isolation and *in vitro* conditions with a self-regulating programme of dedifferentiation, proliferation, pattern formation, differentiation, and plant development.

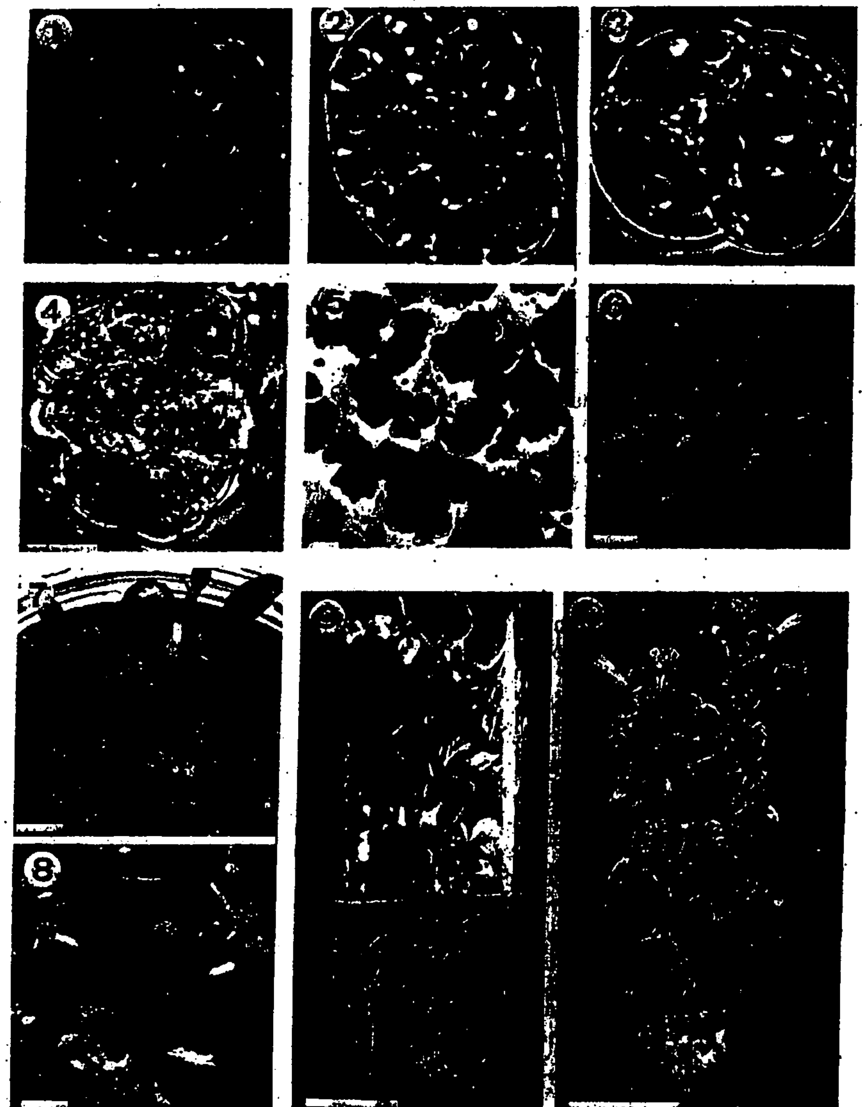
This "*in vitro* competence" probably has a genetic, developmental, and physiological basis. We have at present no way to identify competent cells before they respond. Competence can apparently be lost during isolation and culture of protoplasts. We feel that protoplasts which lack competence when taken into culture cannot be forced to aquire it by *in vitro* manipulation. Competence for sustained division does not automatically include competence for differentiation and plant regeneration and there are numerous cultures and even plants which yield proliferating protoplasts from which no differentiation occurs.

The phenomenon of totipotency of somatic plant tissues and isolated cells is well documented. There is, however, no proof for the hypothesis that *all* plant cells are totipotent and it may be naive to expect that this is the case. It is easy to prove that plant cells are totipotent by culturing the protoplast through to a plant. It is, however, impossible to prove that a specific plant cell is not totipotent. Experience suggests that totipotency may not be a general character of plant cells, but that it is restricted to competent cells.

Plant Species and Genotype

There are plant species (and even genera and families), e.g., *Nicotiana tabacum* (*Nicotiana*, *Solanaceae*), where it has been relatively easy to establish conditions for protoplast proliferation and plant regeneration

[1] W. Halperin, *Annu. Rev. Plant Physiol.* **20**, 395 (1969).



FIGS. 1–10. Developmental stages from isolated protoplasts to plant in *Petunia hybrida*. (1) Freshly isolated leaf protoplast; (2) cell wall regeneration and dedifferentiation at day 2; (3) first cell division at day 4; (4) 50 cell colony at day 10; (5) population of developing clones at the 500 cell stage, 4 weeks; (6) clones on agar solidified medium, 8 weeks; (7) differentiation of shoots (high cytokinin, low auxin); (8) differentiation of roots (no cytokinin, low auxin); (9) protoplast derived plantlet with roots in test tube, 3 months; (10) flowering plant in soil, 6 months. Bar lines: Figs. 1–4: 10 μm; Fig. 5: 100 μm; Figs. 6–9: 1 cm; Fig. 10: 10 cm. Figures are from I. Potrykus, *in* "Yeast, Mould and Plant Protoplasts" (J. R. Villanueva, I. Garcia-Acha, S. Gascon, and F. Uruburu, eds.), p. 319. Academic Press, New York, 1973.

from numerous genotypes with protoplasts from different source tissues, and where these results can be reproduced in independent laboratories. There are, however, also complete plant families, e.g., the *Gramineae* (grasses) where intensive efforts with hundreds of genotypes of many species have never produced clear cut evidence for regeneration of a single plant from a protoplast, and where even the induction of protoplast division is still a severe problem.

Between these two extremes there are very closely related genotypes of the same species, e.g., *Petunia hybrida*, where one can routinely regenerate plants from protoplast-derived clones of one genotype and not, so far, from the other. Clear genetic data on this phenomenon are missing and the way out with a nonresponding genotype has been, so far, not so much through variation of culture conditions but rather through screening for responsive genotypes. Genotype screening for *in vitro* response may pay off in cases where it is not important which genotype is used. Where one must work with a specific genotype, as for example in plant breeding, this option is not available, and one must work on improving other variables.

Source Tissues from Organized Plants and from Cell Culture

Protoplasts are isolated from organized plants and from organized or unorganized cell cultures.

Plants

Developmental state: For plants grown in soil under natural conditions young plants with expanding leaves before the differentiation of flowers generally give best results. As soon as plants begin to flower, it is difficult, if not impossible, to isolate proliferating protoplasts. The same holds true for organs where senescence has started. One generally therefore uses organs before they have fully matured, e.g., expanding leaves at one-half to two-thirds of their final size. Seedling tissues are becoming more commonly used as a source material. Embryonic or meristematic tissue, or complete embryos, may provide protoplasts with the best potential for proliferation; they are, however, mostly difficult to isolate and

the isolation procedures required (high osmotic pressure, high enzyme concentrations, and long incubation times) may in part interfere with this potential.

Leaf: Leaves are the routine source if no specific reasons make it necessary to use other organs. The reason is that the protoplasts are generally easy to isolate and to handle experimentally and it is usually no problem to isolate very large populations of protoplasts at a similar state of differentiation which will proliferate at a high plating efficiency and regenerate plants.

Other differentiated tissues: Parenchymatic protoplasts with small plastids are isolated from stem, petiole, or differentiating root tissues and from the epidermis and vascular parenchyma of leaves. It is possible to culture these protoplasts as well. The greater effort required and the lower plating efficiencies obtained are justified, however, only in cases where specific problems require it. This is also true for the culture of the intensively colored protoplasts isolated from flower petals.

Seedlings: These may provide a convenient source of proliferating protoplasts in cases where attempts to culture protoplasts from leaves have failed. Protoplasts from total seedlings, as well as from hypocotyl, cotyledons, and from seedling roots have been successfully cultured. The disadvantage of limited material may be well compensated for by a proliferation response unobtainable from other organs.

Embryos: These yield populations of tiny protoplasts as long as they are immature. However, isolation is so tedious and culture so difficult that we cannot recommend this source unless it is absolutely necessary. Meristems from the shoot apex or root tips have been used as source in exceptional cases but are not a tissue to be generally recommended because of reasons similar to those for embryos.

Shoot cultures: Axenic shoot cultures proliferating under *in vitro* conditions are the source material of choice in species where they can be easily established and maintained and many successful protoplast laboratories are using them nearly exclusively. There are two types: one is based on normal developed shoots which are rooted and proliferated by rooting shoots under sterile conditions (Nagy and Maliga,[2] as described in protocol 1); the other is based on multiple adventitious shoots proliferating from a wound callus at the base of a shoot grown on a culture medium with growth regulators (as exemplified by Bindings' work).[3] Shoot cultures have been established from numerous herbaceous dicots, but not so far from any graminaceous species. Protoplasts from shoot cultures are very easy to isolate and have a high proliferation and regeneration potential.

[2] J. I. Nagy and P. Maliga, *Z. Pflanzenphysiol.* **78**, 453 (1976).
[3] H. Binding, *Physiol. Plant.* **35**, 225 (1975).

Cell Cultures

Organized cell cultures: Embryogenic cultures are becoming available in an increasing number of plant species, including as interesting groups as the cereals and legumes.[4] The characteristic of these cultures is that small groups of cell have the potential to differentiate to embryos. Differentiation is suppressed by a synthetic growth regulator which at the same time enhances proliferation. An embryogenic suspension can be considered an ideal source for totipotent protoplasts and this potential is routinely available in a system such as, for example, carrot. In cereals it has still to be convincingly demonstrated that the embryogenic potential is passed through the single cell state.

Unorganized cell cultures: Cultures capable of regeneration: In several plant species it is possible to establish dedifferentiated cell cultures by enhancing and maintaining the wound response by synthetic growth regulators, with little interference with the morphogenic potential of the cells in prolonged culture (e.g., *N. tabacum and D. carota*). These cultures can be grown on agar-solidified culture media as callus cultures, or can be dissociated into fine suspensions in liquid on shakers. Such cultures, from a few suitable genotypes, and with the appropriate treatments, are another convenient source for potentially totipotent protoplasts.

Cell lines: A cell line culture type[5]) has arisen spontaneously in nearly every plant species kept in culture for long periods of time, including many cereals and grain legumes. Such cell lines are characterized by a short cell cycle, proliferation on a single cell basis, friability, and, unfortunately, a complete lack of morphogenic response. Protoplasts can be easily isolated and, in most cases, cultured. There is, however, very little chance of plant regeneration from such protoplasts.

Environmental Conditions

Care of plants: The care of the plants before the isolation of protoplasts can be a key factor. Plants which have been badly treated rarely yield proliferating protoplast populations. Plants should be watered at regular times with the correct amount of water or fertilizer solution. The soil type should be appropriate and its structure should provide good aeration of the root system. Clay pots are to be preferred to plastic ones. The growth conditions should provide a continuously favorable environment and avoid extremes in any aspect.

[4] P. J. Dale, *in* "Protoplasts 1983: Lecture Proceedings" (I. Potrykus, C. T. Harms, A. Hinnen, R. Hutter, P. J. King, and R. D. Shillito, eds.), p. 31. Birkhauser, Basel, 1983.
[5] P. J. King, *in* "Perspectives in Plant Cell and Tissue Culture" (I. K. Vasil, ed.), p. 25. Academic Press, New York, 1980.

Similar recommendations as for soil-grown plants apply for *in vitro* grown shoot cultures.

Season: It is the experience in many laboratories that season can play a crucial role even with optimized systems. This is not only experienced with greenhouse material, where one can expect it, but also with material which is grown in incubators without any direct interaction with climatic factors. Experiments which do not work between November and February may work well when repeated between March and June.

Light: This, of course, plays a crucial role. The greatest amount of data has been accumulated on the effect of light intensity. Generally, protoplasts isolated from plants grown under high light intensity are very sensitive to the isolation procedures and, if they survive, proliferate poorly. Experimental plants have therefore to be kept in low light intensity, i.e., 3000 lux or lower, for at least 1 day, and preferably longer, before the experiments. The effect of the light quality is less obvious, but not negligible. It has, however, not been studied carefully enough to allow clear recommendations. Natural light seems to be superior to artificial; cool fluorescence tubes should be used in mixtures which cover the visible spectrum more homogeneously than one type of tube. The contribution of the short UV spectrum should be small. If mercury vapor lamps are used they should be combined with sodium vapor ones. The photoperiod probably plays a role too; however, there is no comparative study of this parameter in higher plants.

Temperature and relative humidity: These also contribute to the actual physiological state of the cell at the time of isolation, but again there are no clear data to aid the experimenter.

In general it is advisable to grow the plants under conditions close to their natural climate.

Care of cell cultures: Cell culture material can be very sensitive to alterations in the culture conditions, e.g., the time interval of subculture, the dilution ratio with fresh culture medium, the material and geometry of the culture vessel, and the aeration and shaking conditions. Isolation of protoplasts is often possible only during a short time period in the exponential growth phase of the culture and quite often cell cultures respond to unknown alterations in the culture conditions in a way which prevents protoplast isolation totally, or reduces their quality. The only solution in such a case is often to establish a fresh culture. It should, however, be made clear that there are many examples of completely unproblematic cell cultures.

Endogenous Factors

Response to wounding and pest treatments: Mechanical injury can, within hours, alter the physiology of the whole plant in such a way that

protoplast culture is not possible for several days. If, for example, leaves are taken from the same plant over a long period of time, it is recommended that 1 week of recovery be allowed between experiments. This proviso applies for every type of extreme stress situation. If pest treatments are necessary, there should be a similar time interval between treatment and isolation of protoplasts.

Preculture: Excision of organs and preculture on a culture medium to induce dedifferentiation *in situ* prior to protoplast isolation has been helpful with many recalcitrant plants, e.g., legumes. On the other hand, attempts at a similar approach with cereal leaves failed completely because the cell walls were modified, in response to even 1 days preculture, such that protoplast isolation was no longer possible.[6]

Cell cycle phase: The question whether the cell cycle phase plays a role, which phase is optimal for protoplast isolation, and whether actively cycling cells are better than resting ones cannot be answered conclusively. Fast cycling cells can be sensitive to the isolation procedure but cell culture protoplasts are normally isolated from cycling suspension cultures as opposed to resting ones. Protoplasts isolated from fast growing cell suspensions rarely reach the high plating efficiencies of arrested leaf mesophyll protoplasts, but this may be due to other factors than cell cycle phase, such as the state of differentiation.

Sterilization Procedure

The sterilization procedure has to be adapted to the type of organ or tissue used in order to ensure that the majority of the cells are not already killed or injured by the sterilizing agent. The minimum concentration and the minimum time required for safe sterilization has to be established for every case. There are also specific sensitivities of tissues to an alcohol pretreatment or to specific sterilizing agents which have to be taken into account. One of the advantages of sterile *in vitro* shoot cultures is the fact that there is no danger of oversterilization or incomplete removal of the sterilizing agent.

Mechanical Treatments Prior to Enzyme Application

The outer cell layer of plants has evolved to prevent access to the inner cell layers. The cuticle, a complex layer of polymers excreted to protect the cells, is not penetrable by the enzymes used for protoplast isolation. It can be degraded by β-glucuronidase, but the enzymes available are toxic and it is difficult to control their action. The routine procedure is therefore to mechanically bypass the epidermal cell layer by either peeling off the epidermis (where possible), brushing with an abrasive, or

[6] I. Potrykus, unpublished (1974).

slicing the organ into cross sections. These methods require some care and practice to avoid excessive damage of the inner cell layers.

Plasmolysing Conditions

Plant cells have an internal pressure (turgor pressure) which is contained by the rigid cell wall. Digestion of the wall in a hypotonic environment would cause the protoplasts to burst. Isolation is therefore carried out in hypertonic conditions which leads to plasmolysis and frees the cell wall from its structural role. Both the agent used for plasmolysing the cells and the intensity of the osmotic pressure used can be critical. This is particularly important for embryonic or meristematic cells which require high osmotic pressures and a long incubation time. Our laboratory routinely uses mixtures of mannitol and calcium chloride in the range of 400 to 800 mOsm/kg H_2O. There are, however, cases where these conditions are not successful and where variations in type and concentration of osmotica have to be screened.

Enzyme Treatment

Plant cell walls are complex chemical and physical structures of celluloses, hemicelluloses, pectins, proteins, and other polymers. Enzymes for protoplast isolation are commercially available. These are usually obtained from culture filtrates of microorganisms (mostly fungi) which normally grow on organic matter in forests. The enzymes applied are therefore mixtures of cellulases, hemicellulases, and pectinases. These commercial enzyme preparations, unfortunately, also contain nucleases, proteases, lipases, phosphatases, and other undesired enzymes. Separation of individual enzymes and application of pure enzymes and controlled mixtures of sequences has been possible[7] but is not required in most cases and is therefore not routine. Even purification from low-molecular-weight substances is not required in numerous cases where plating efficiencies higher than 90% can be obtained with unpurified enzymes. However, in more sensitive systems extra purification (by gel filtration) may be essential.

The standard treatment is with a mixture of a "cellulase" and a "pectinase" at concentrations around 0.5–2% w/v dissolved in an osmoticum (giving 400–800 mOsm/kg H_2O) at pH 5.2–5.6, 24° for a few hours. There are probably as many variations as laboratories, plants, and types of source material and we can discuss a few general points only.

Be flexible. The cell walls of plant tissues vary with the environmental conditions and growth conditions. Routine established standard protocols

[7] S. Ishii and Y. Mogi, *in* "Protoplasts 1983: Poster Proceedings" (I. Potrykus, C. T. Harms, A. Hinnen, R. Hutter, P. J. King, and R. D. Shillito, eds.), p. 6. Birkhauser, Basel, 1983.

may work perfectly in one laboratory, but fail even if reproduced as precisely as possible in a different laboratory.

The incubation time required for protoplast release depends on the actual state of the source tissue, the enzyme type and mixture, the enzyme concentration, the pH, the osmoticum and osmotic pressure, the incubation temperature, the ratio of tissue : enzyme volume, and the mechanical conditions during incubation.

We have discussed source tissue above. A few comments should be made concerning the other parameters. The fastest isolation procedure is not necessarily the best if related to later plating efficiency of the protoplasts. Efficient enzyme types can be toxic (e.g., pectolyase Y23 or helicase); the optimum mixture has to be established by screening. Embryonic or meristematic tissues require more hemicellulases and pectinases.

Enzyme concentrations higher than 2% w/v are often deleterious although they reduce the time of incubation. Our laboratory avoids total concentrations above 4% and mixtures of more than 3 commercial enzymes. The pH optimum of nearly all available enzymes is around pH 4. Plant cells do not normally tolerate pH values below 5.2. Higher pH values and longer incubation times may be necessary with sensitive species.

Mannitol as major osmoticum is sufficient in many cases but there should be always some calcium present (chloride: see protocols) in order to stabilize the plasma membrane. There appears to be no advantage to be gained in using complex osmotica. The osmotic pressure should be as low as possible, but high enough to reduce spontaneous fusion of adjacent cells during isolation.

The incubation temperature should not be raised above 35°. Sensitive systems may benefit from long-term isolations at low temperatures (7–12°). Difficult systems may respond to sequences of high and low temperatures. The incubation of too much tissue in too little enzyme solution has spoiled many experiments. We recommend that not more than 1 g of tissue be incubated in 10 ml of enzyme solution. It is often essential to incubate under conditions with a large ratio of surface to depth (e.g., 10 ml in a 9-cm petri dish).

Cell culture protoplasts tolerate mechanical shaking during isolation, and in many cases even require this help. There is good experience using roller bottles, and with circular shaking of petri dishes on a rocking table or a rotatory shaker. We cannot recommend mechanical shaking during isolation of chloroplast-containing cells. Gentle shaking by hand, when the incubation is nearly completed is, however, helpful to maximize protoplast release and shorten the incubation time. Vacuum infiltration of the enzyme solution into the tissue to replace the intercellular air shortens

incubation time and improves considerably the homogeneity and quality of protoplast populations isolated from differentiated tissue. With this treatment the danger lies in the application of a too strong vacuum for too long a time. Finally it should be mentioned that preplasmolysis for 0.5–1 hr in osmoticum before incubation in enzyme solution can be beneficial.

It requires some experience to recognize, by microscopic examination, the time point when the incubation should be terminated. Protoplasts can be sensitive to overdigestion as well as to underdigestion. Few protoplasts can recover when the plasma membrane (the plasmelemma) has been disrupted, and "partial protoplasts" having patches of residual cell wall develop far worse than complete protoplasts.

Protoplast Harvest and Purification

Protoplasts are easily separated from incomplete digested tissues by sieving through meshes with pore sizes adjusted to the size of the protoplasts. Both washing and purification depend upon differences in the relative buoyant density of protoplasts and washing solution. Sugar and sugar alcohols (e.g., sucrose and mannitol) have relatively high buoyant densities. Salt solutions (e.g., $CaCl_2$ and $MgCl_2$) have relatively low buoyant densities at the same osmotic pressure. A practical nonagram giving the relation between osmalility, molality, and buoyant density has been published.[8] Chloroplast-containing leaf protoplasts have high buoyant densities, cytoplasmic protoplasts have medium, and vacuolated parenchymatic protoplasts with small plastids have low buoyant densities. Washing and collection by sedimentation require the combination of protoplasts with a higher buyoant density with washing solutions with relatively low buoyant densities. For flotation of protoplasts the opposite applies. Care must be taken to maintain a similar osmotic pressure throughout the procedures and a convenient osmometer is of great help. Protoplasts should be washed at least three times by repeated sedimentation or flotation and resuspension. A flotation step may be required for purification in cases where the protoplasts are contaminated with debris which does not separate out during the sieving step. If this is not possible then swirling of protoplasts sedimenting in a low density osmoticum in petri dishes helps to collect the perfectly spherical protoplasts in the center. Step gradients have been described which allow not only purification but also fractionation of protoplast populations into fractions of different buoyant densities, which may correlate with their capacity for proliferation. Gradients of osmotically inactive substances such as Percoll[9] in combination with osmotica of low buoyant density may yield clean populations where simple osmotica alone fail. Finally, automated cell sorting is also possible with protoplasts, although this is not yet sufficiently developed to be in routine use.

[8] C. T. Harms and I. Potrykus, *Theor. Appl. Genet.* 53, 57 (1978).

Careful separation of protoplasts from the enzymes used for isolation is essential, as resynthesis of a cell wall, which is inhibited by residual enzymes, is normally an absolute prerequisite for proliferation.

Protoplast Culture

Assuming that there was no important mistake in the procedure so far, there are numerous further factors which could interfere with or promote the capacity of the isolated protoplast to dedifferentiate and to proliferate. Among these are the population density, the composition of the culture medium, particularly growth regulators, the osmotic pressure, physical culture conditions, and undefined factors in the culture medium.

Population density: This is a clear case and can be tested easily. Protoplast populations, with exceptions, express a clear optimum of plating efficiency with regard to population density, and protoplasts do not divide at all outside a specific population density range. It is possible to grow optimized systems down to very low population densities and individual single protoplasts in micro drops[10] which suggests that the ratio of protoplasts to volume of culture medium is important. The usual range of protoplast population densities is between 2×10^4 and 2×10^5/ml, increasing with decreasing protoplast size and vice versa.

Culture medium: The composition of the culture medium plays an important role. This typically contains ions of inorganic salts, vitamins, a carbon and energy source (sucrose or glucose), growth regulators, and an osmostabilizer (see the table). No protoplast culture medium has been completely optimized by checking the effect of variations in every single component against every other component; this is also not required, as plating efficiencies of higher than 90% are obtained using partially optimized media. There are efficient screening techniques available such as the "Microdrop-Array Technique"[11,12] which can be used to study the effect of systematic variations of culture medium factors. If changes in population density and the established culture media fail to induce sustained divisions the first parameters to vary are the growth regulators. With the culture medium giving best survival and a fixed population den-

[9] W. Wernicke, H. Loerz, and E. Thomas, *Plant Sci. Lett.* 15, 239 (1979).
[10] K. N. Kao, *Mol. Gen. Genet.* 150, 225 (1977).
[11] I. Potrykus, C. T. Harms, and H. Loerz, *Plant Sci. Lett.* 14, 231 (1979a).
[12] C. T. Harms, H. Loerz, and I. Potrykus, *Plant Sci. Lett.* 14, 237 (1979).

sity of 2–5 × 10^4/ml, a combination of a dilution series of an auxin versus a cytokinin both in the range between 0.05 and 5 mg/liter should be tested, followed by similar combinations with other types of auxins and cytokinins used in the first screen. Combinations of different auxins (e.g., 2,4-D and NAA) may help. It may be necessary to reduce the hormone concentrations by up to 10-fold after a few days of culture.

The physical culture conditions are a further important parameter. Most frequently "liquid thin layer" cultures in petri dishes will do. It is, however, important to make the layer of protoplast suspension as thin as possible (e.g., 0.7 ml in a 3.5-cm petri dish; 2 ml in a 6-cm petri dish). The depth of the liquid layer alone can inhibit proliferation. Some protoplasts develop only if plated into a gel, others do not develop under these conditions. Agar as gelling agent may suffice with insensitive protoplast types, but agarose gives better plating efficiencies and enables development of sensitive protoplasts. The "bead-type" culture technique where segments of protoplast containing gels are floated in larger volumes of liquid culture medium and aerated on a rotary shaker has considerably improved the plating efficiency in many systems and has enabled sustained development in systems where protoplasts did not previously divide.[13]

Microdrops of 20–40 μl are helpful if the total number of protoplasts is limited.[10] A culture medium with a high buoyant density which maintains the protoplasts floating during the critical initial culture stages, or a floating membrane or filter support onto which the protoplasts are plated are further possible variations.

Osmotic pressure: An incorrect osmotic pressure of the culture medium can be sufficient to inhibit development. It is important to check this early in the experiments by culture in a series of different osmotic values down to ~300 mOsm/kg H_2O, and to recheck its effect with altered conditions. Unfortunately, not only the quantity of the osmostabilizer but also its quality are important and there are even interacting effects of the type of osmoticum used during isolation and the type of culture medium used for subsequent culture. The relatively high osmotic pressure of the initial culture medium should be gradually reduced to lower and lower levels as the culture develops.

Environmental conditions: Of the environmental factors temperature and light are most important. A continuous temperature of 24–26° is recommended as a baseline. There are, however, protoplasts which will not divide unless cultured at 28° or higher, some of which will not develop at this temperature, and others which require a lower temperature. An initial phase of 12–24 hr at 12° has been found to promote subsequent development at 24° in some cases. Light is normally not required for induction of divisions, although it may enhance further development. The light intensity should not exceed 1000 lux, as high intensities inhibit early development. Fluorescence tubes are a suitable light source. The light quality plays a role as has been found in experiments with monochromatic light.[6] There are, however, no clear and conclusive data available.

The choice of container in which to culture the protoplasts can have profound effects. Plasticware is now used in most laboratories and a range of petri dishes from different suppliers should be used. We have ourselves found that some brands (e.g., Falcon) can be deleterious under our conditions to even robust protoplasts such as those from *N. tabacum*.

Undefined factors: There is a further group of factors to be tested if all the variations suggested above do not succeed: conditioned medium, nurse cultures and feeder layers, plant extracts, and X-plates.

Conditioned medium is harvested from actively dividing cell suspensions and added to culture media in different proportions. The time point for the harvest is critical and recommended to be during the early log phase of growth.

Nurse cultures make use of diffusible substances released from cell cultures into the surrounding culture medium by coculturing protoplasts and cell cultures. The growth of the cell cultures in this combination may be inhibited by the high osmotic pressure required for the protoplasts. Feeder layers consist of protoplasts or cells plated separately in an independent layer (usually below the protoplast layer); often the cells of the feeder layer are protoplasts with high division potential which have been inhibited prior to plating by X-irradiation.[14] Another approach to feeding is the use of competent auxotrophic protoplasts which are mixed and plated with the nondividing protoplasts and later selected against by nonpermissive conditions.[15]

X-plates, where a petri dish is separated into quadrants by permeable bars and protoplasts are plated alternatively with culture medium with a lower osmotic potential[16] or containing activated charcoal,[17] has been reported to improve or enable protoplast division. The authors have no personal experience with this culture technique.

[13] R. D. Shillito, J. Paszkowski, and I. Potrykus, *Plant Cell Rep.* **2**, 44 (1983).

[14] D. Raveh, B. Huberman, and E. Galun, *In Vitro* **9**, 216 (1973).

[15] T. Hein and O. Schieder, *in* "Protoplasts 1983: Poster Proceedings" (I. Potrykus, C. T. Harms, A. Hinnen, R. Hutter, P. J. King, and R. D. Shillito, eds.), p. 104. Birkhauser, Basel, 1983.

[16] J. F. Shepard, *in* "Genetic Improvement of Crops: Emergent Techniques" (I. Rubenstein, B. Regenbach, R. L. Phillips, and C. E. Green, eds.), p. 185. Univ. of Minnesota Press, Minneapolis, 1980.

[17] I. Carlberg, K. Glimelius, and T. Eriksson, *in* "Protoplasts 1983: Poster Proceedings" (I. Potrykus, C. T. Harms, A. Hinnen, R. Hutter, P. J. King, and R. D. Shillito, eds.), p. 258. Birkhauser, Basel, 1983.

MEDIA COMPOSITION[a]

Inorganic salts used in tissue culture media

Medium	T[b]	LS[c]	K3[d]	H/J/KO[e]	CS[f]	CP	CPW[g]	B5P[h]	W and B[i]	CC[j]
Macro elements[k] (mg/ml final concentration)										
KCl									910	
KNO_3	950	1900	2500	1900	1900	950	101	1900	80	1212
KH_2PO_4	68	170		170	170	680	27	170		136
$NaNO_3$									1800	
NH_4NO_3	720	1650	250	600	1650	825		600		640
$NaH_2PO_4 \cdot H_2O$			150						300	
$CaCl_2 \cdot 2H_2O$	220	440	900	600	440	438	1480	600		588
$MgSO_4 \cdot 7H_2O$	185	370	250	300	370	123	240	300	1360	247
$(NH_4)_2SO_4$			134						790	
$Ca(NO_3)_2 \cdot 4H_2O$									200	
$NaSO_4$									88	
Micro elements[k] (mg/liter final concentration)										
Na_2EDTA	74.6	74.6	74.6	74.6	74.6	74.6		74.6		37.3
$FeSO_4 \cdot 7H_2O$										27.8
$FeCl_3 \cdot 6H_2O$	27.0	27.0	27.0	27.0	27.0	27.0		27.0	4.15	
H_3BO_3	10.0	6.2	3.0	3.0	6.2	6.2		3.2	1.5	3.1
KI		0.83	0.75	0.75	0.83	0.83		0.415	0.75	0.83
$MnCl_4 \cdot 4H_2O$									4.5	
$MnSO_4 \cdot H_2O$	17.25	16.9	10.0	10.0	22.3	16.9		8.45		11.15
$ZnSO_4 \cdot 7H_2O$	10.0	8.6	2.0	2.0	10.6	8.6		4.3	1.5	5.76
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025	0.025	0.025	0.025		0.013	0.013	0.025
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25		0.125		0.24
H_2Mo_4									0.002	
$CoCl_2 \cdot 6H_2O$			0.025	0.025	0.025					
$CoSO_4 \cdot 7H_2O$		0.03				0.03	0.025	0.015		0.028

Vitamins and other organics used in culture media (mg/liter concentration)

Medium	T	LS	K3	H/J[l,m,n]	KO[l,m]	CS	CP	B5P	W and B	CC
m-Inositol	100.00	100.00	100.00	100.00	100.00	100.00	100.00	1000	100	90
Vitamins[e]										
Biotin	0.05			0.01	0.01		0.01			
Pyridoxine–HCl	0.50		0.10	1.00	1.00	0.50	1.00	0.1	0.1	1.0
Thyamin–HCl	0.50	0.04	1.00	10.00	10.00	0.10	10.00	1.0	0.1	8.5
Nicotinamide				1.00	1.00		1.00			
Nicotinic acid	5.00		0.10			0.50		0.1	0.5	6.0
Folic acid	0.50			0.40	0.40		0.40			
D-Ca-pantothenate				1.00	1.00		1.00			
p-Aminobenzoic acid				0.02	0.02		0.02			
Choline chloride				1.00	1.00		1.00			
Riboflavin				0.20	0.20		0.20			
Ascorbic acid				2.00	2.00		2.00			
Vitamin A				0.01			0.01			
Vitamin D_3				0.01	0.01		0.01			
Vitamin B_{12}				0.02	0.02		0.02			
Glycine	2.00			0.10					3.0	2.0
Coconut water (%v/v)										10.0

(continued)

TABLE (*continued*)

Medium	T	LS	K3A	K3C	K3B	H	J	K0	CP	CS	CPW13M	CPW21S	B5P	W and B	CC
	Carbohydrates and phytohormones														
Sugars and sugar alcohols (g/liter)															
Sucrose	10.0	30.0	102.96	102.96	36.0	0.25	0.25		20.0	20.0		210	30.0	20.0	20.0
Glucose						68.40	21.0	68.4							
Mannitol						0.25	0.25	0.25	76.5		130		70.0		36.43
Sorbitol						0.25	0.25	0.25							
Cellobiose						0.25	0.25	0.25							
Fructose						0.25	0.25	0.25							
Mannose						0.25	0.25	0.25							
Rhamnose						0.25	0.25	0.25							
Ribose						0.25	0.25	0.25							
Xylose			0.25	0.25	0.25	0.25	0.25	0.25							
Hormones (mg/liter)															
2,4-D		0.05	0.10	0.05	0.05	0.10		2.0		0.4			0.02		2.0
p-CPA									4.0						
NAA		2.00	1.00	2.00	2.00	1.00	1.0						1.0	0.5	
BAP		1.10	0.20	0.10	0.10	0.20	0.25	0.5							
Kinetin		0.10		0.10	0.10				0.6	0.04					
Zeatin													0.5	0.5	
Final pH	5.5	5.8	5.8	5.8	5.8	5.8	5.8	6.0	5.8	5.7	5.6	5.6	5.7	5.5	5.5

[a] Where the inorganic component is common, i.e., K3 and CPW media and H/J/K0, these have been given together.
[b] J. P. Nitsch and C. Nitsch, *Science* 163, 85 (1969).
[c] E. M. Linsmaier and F. Skoog, *Physiol. Plant.* 18, 100 (1965).
[d] J. I. Nagy and P. Maliga, *Z. Pflanzenphysiol.* 78, 453 (1976).
[e] K. N. Kao and M. R. Michayluk, *Planta* 126, 105 (1975).
[f] T. Murashige and F. Skoog, *Physiol. Plant.* 15, 473 (1962).
[g] Z.-H. Xhu, M. R. Davey, and E. C. Cocking, *Z. Pflanzenphysiol.* 104, 289 (1981).
[h] O. L. Gamborg, R. A. Miller, and K. Ohyama, *Exp. Cell Res.* 50, 151 (1968).
[i] H. N. Wood and A. C. Braun, *Proc. Natl. Acad. Sci. U.S.A.* 47, 1907 (1961).
[j] I. Potrykus, C. T. Harms, and H. Loerz, *Theor. Appl. Genet.* 54, 209 (1979).
[k] Macroelements are usually made up as a 10× concentrated stock solution, and microelements are a 1000× concentrated stock solution.
[l] Citric, fumaric, and malic acid (each 40 mg/liter final conc.) and sodium pyruvate (20 mg/liter) are prepared as a 100× concentrated stock solution, adjusted to pH 6.5 with NH_4OH, and added to these media.
[m] Adenine (0.1 mg/liter), and guanine, thymine, uracil, hypoxanthine, and cytosine (0.03 mg/liter) are prepared as a 1000× concentrated stock solution, adjusted to pH 6.5 as above and added to these media.
[n] The following amino acids are added to this medium using a 10× stock solution (pH 6.5 with NH_4OH) to yield the given final concentrations: glutamine (5.6 mg/liter), alanine, glutamic acid (0.6 mg/liter), cysteine (0.2 mg/liter) asparagine, aspartic acid, cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine (0.1 mg/liter).
[o] Vitamin stock solution is normally prepared 100× concentrated.

Finally, extracts of various kinds may help if everything else has failed. Coconut water, leaf extracts, extracts from meristematic cultures, and xylem exudate have improved response in early phases of studies and could later be replaced by completely defined culture media.

Plant Regeneration

Plant regeneration from cell cultures derived from complex explants may present a completely different problem to plant regeneration from protoplast-derived cultures. Explant-derived cell cultures may, and often do, contain adventitious meristems which proliferate as meristems. Regeneration is then not more than a release of the morphogenic potential of the preexisting meristems by removal of some sort of repression. Protoplast-derived cell clones have to build new meristems in a mass of dedifferentiated cells and there is no guarantee that cells have this potential and are able to maintain it over many subcultures, even if it is possible to regenerate plants from complex explants. Fortunately, many genotypes of herbaceous plant species have this inherent capacity (competence for plant regeneration from isolated single cells). In those cases where the cells are competent, plant regeneration is easy, and with the best model plants also very efficient. A relative increase in cytokinins and decrease in auxins in the culture medium, and the presence of light, are the basic conditions which allow the self-regulating process of shoot meristem induction, maturation, and shoot differentiation to proceed. Low levels of auxins in the absence of cytokinins in the culture medium favor the development of roots. In a few cases conditions have been discovered which allow the development of somatic embryos and their later maturation and germination.[18] In cases where the inherent competence is missing we are, to date, helpless.

Concluding Remarks

Our interpretation of the biological phenomenon of sustained development and plant regeneration from isolated protoplasts is that competent cells have a self-regulatory program to undergo dedifferentiation, sustained mitotic divisions, and differentiation. This program is triggered by a "wound response" initiated via isolation and amplified by hormone treatments. The experimenter helps by providing nutritional and environmental conditions which allow this process to proceed. This works with competent cells and does not work with noncompetent cells. Formation of shoot and root meristems from protoplast-derived cultures depends again on this biological phenomenon of competence, which we can observe, but not define.

[18] L. Li and H. W. Kohlenbach, *Plant Cell Rep.* 1, 209 (1982).

Protocols for the Preparation and Culture of Protoplasts and Regeneration of Plants

We describe four protocols which are in everyday use in our laboratory and one which has been described in the literature.

A number of things are common to these protocols.

Centrifugations are carried out at 60 g (100 g in protocol 3) except where otherwise stated.

Washing solutions (osmoticum) in protocols 1, 2, 4, and 5 are buffered with 0.5% w/v 2-(*N*-morpholino)ethanesulfonic acid (MES) and adjusted to pH with KOH except where otherwise stated.

Counting of protoplasts: counting of protoplasts is carried out by placing a drop of a 1 : 10 dilution of the suspension in calcium chloride in a hemocytometer, counting, and estimating the density in the original suspension.

Abbreviations

MES: 2-(*N*-morpholino)ethanesulfonic acid
NAA: naphthaleneacetic acid
2,4-D: 2,4-dichlorophenoxyacetic acid
p-CPA: p-chlorophenoxyacetic acid
BAP: 6-benzylaminopurine

Materials

Sources for the plant material are given with each individual protocol.

Table top centrifuge: Universal 2 (Hettich Centrifugen, 72, Tuttlingen, West Germany).

Osmometer: Roebling Micro-Osmometer (Infochroma AG., Baarerstrasse 57, CH-6300, Zug, Switzerland).

Rocking table: Heidolph Reax 3 rocking table (Salvis AG., CH-6015, Reussbuehl, Switzerland).

Stainless steel sieves: Saulas and Co. (St. Louis, Paris 10, France). The 10-cm-diameter containers used for the "bead-type" culture are obtained from Semadani AG (Ostermundigen, CH-3072, Switzerland.)

Petri dishes: these are obtained from a range of suppliers but we have found that those from some suppliers can be toxic to some protoplast types.

SeaPlaque agarose: Marine Colloids, FMC Corp. (5 Maple Street, Rockland, Maine 04841).

Cleaned agar: this is prepared by washing with water, acetone, and ethanol in succession.[13,19]
Tween 80: ICI (Runcorn, England) or Merck-Schuchardt (Hohenbrunn, Munchen, West Germany).
Percoll: Pharmacia (Uppsala, Sweden).
Greenzit, Ciba-Geigy AG (Basel, Switzerland).
Cellulase "Onozuka" R10 and Macerozyme R10: Yakult Pharmaceutical Ind. Co. Ltd. (Shingikan Cho, Nishinomiya, Japan).
Meicelase: Meiji Seika Kaisha Ltd. (Tokyo, Japan).
Driselase: Chemische Fabrik Schweizerhalle (CH-4013, Basel, Switzerland).
Hemicellulase: Sigma Chemical Co. (P.O. Box 4508, St. Louis, Mo. 63178).
Rhozyme HP150: Roehm and Haas Co. (Philadelphia, Penn.).
Pectinol: Roehm GmbH Chemische Fabrik (Darmstadt, West Germany).
All other organic and inorganic substances were of the highest purity available from the usual commercial sources.

1. *Preparation and Culture of Protoplasts from a Sterile Shoot Culture, and Regeneration of Plants*

The example given is for protoplasts from shoot cultures of the widely used genotype of *N. tabacum* cv. Petit Havana, SR1.[20] This material is grown as sterile axenic shoot cultures.

The protocol for protoplast isolation is modified from that of Nagy and Maliga[2] and works equally well with tobacco leaf material from greenhouse grown material. In the latter case the leaves must first be surface sterilized (see protocol 2) and then either sliced or the lower epidermis removed by peeling. The culture method uses complex media based on that of Kao and Michayluk[21] (see the table) and the agarose "bead-type" system described by Shillito *et al.*[13] to obtain high division frequencies and rates of conversion of protoplasts to colonies.

Colonies are transferred to agar-solidified medium for 1 subculture and then placed on regeneration medium to promote the formation of shoots. Regenerated shoots are cultured on the original shoot culture medium.

Source of Material. Shoot cultures are established from seed which is sterilized using mercury chloride (see protocol 2) or sodium hypochlorite (5 min, 1.4% w/v containing 0.05% w/v Tween 80) and plants arising are

[19] A. C. Braun and H. N. Wood, *Proc. Natl. Acad. Sci. U.S.A.* **48**, 1776 (1962).
[20] P. Maliga, A. Sz. Breznovits, and L. Marton, *Nature (London), New Biol.* **244**, 29 (1980).
[21] K. N. Kao and M. R. Michayluk, *Planta* **126**, 105 (1975).

serially subcultured every 6 weeks as cuttings on T medium[22] (see the table) solidified with 0.8% w/v cleaned agar at 26° in 16 hr/day light (1000–2000 lux) in a growth chamber.

Preparation of Protoplasts. Just fully expanded leaves of 6-week-old shoot cultures are removed under sterile conditions and wet thoroughly with enzyme solution. The leaves are then cut into 1- to 2-cm squares and floated on enzyme solution (1.25% w/v Cellulase "Onozuka" R10, 0.4% w/v Macerozyme R10 in K3A medium with 0.4 *M* sucrose) in petri dishes (~1 g leaves in 12 ml enzyme solution in a 9-cm diameter petri dish). These are sealed and incubated overnight at 26° in the dark.

The digest is gently agitated and then left for a further half hour to complete digestion. The solution is filtered through a 100-μm stainless-steel mesh sieve and washed through with one-half volume of 0.6 *M* sucrose (MES, pH 5.6), distributed into capped centrifuge tubes, and centrifuged for 10 min.

The protoplasts collect at the upper surface of the medium. The medium is then removed from under the protoplasts. A simple method of doing this uses a sterilized canula (A. R. Howell Ltd., Kilburn High Rd., London NW6, England) attached to a 60-ml disposable plastic syringe. This must be done slowly so as to avoid disturbing the layer of protoplasts excessively. Alternatively, the protoplasts can be collected using a pipette (with a medium orifice).

The protoplasts are resuspended in K3A medium (see the table) containing 0.4 *M* sucrose. Washing of the protoplasts is carried out by repeated (3×) flotation and replacing of the medium in this way. A sample is taken for counting before the last centrifugation, and the protoplasts resuspended the last time in H medium (see the table), at a concentration of 10^5 to 2×10^5/ml.

Alternatively the protoplasts can be resuspended in K3A or other media or osmoticum and manipulations such as transformation or fusion carried out (e.g., as described by Paszkowski and Saul[23]).

Culture. The protoplast suspension in H medium is pipetted into petri dishes (1.5 ml/6-cm-diameter petri dish, respectively) and an equal volume of liquified K3A medium (0.3 *M* sucrose, Nagy and Maliga,[2] see the table) containing 1.2% w/v SeaPlaque agarose, cooled to 40°, is added. The protoplasts are distributed evenly by gentle swirling, and the medium allowed to gel.

Petri dishes are sealed with Parafilm or an equivalent and incubated in the dark at 26° overnight, followed by transfer to the light (500 lux) at the same temperature for a further 6 days.

[22] J. P. Nitsch and C. Nitsch, *Science* **163**, 85 (1969).
[23] J. Paszkowski and M. W. Saul, this volume [28].

Cleaned agar: this is prepared by washing with water, acetone, and ethanol in succession.[13,19]

Tween 80: ICI (Runcorn, England) or Merck-Schuchardt (Hohenbrunn, Munchen, West Germany).

Percoll: Pharmacia (Uppsala, Sweden).

Greenzit, Ciba-Geigy AG (Basel, Switzerland).

Cellulase "Onozuka" R10 and Macerozyme R10: Yakult Pharmaceutical Ind. Co. Ltd. (Shingikan Cho, Nishinomiya, Japan).

Meicelase: Meiji Seika Kaisha Ltd. (Tokyo, Japan).

Driselase: Chemische Fabrik Schweizerhalle (CH-4013, Basel, Switzerland).

Hemicellulase: Sigma Chemical Co. (P.O. Box 4508, St. Louis, Mo. 63178).

Rhozyme HP150: Roehm and Haas Co. (Philadelphia, Penn.).

Pectinol: Roehm GmbH Chemische Fabrik (Darmstadt, West Germany).

All other organic and inorganic substances were of the highest purity available from the usual commercial sources.

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serially subcultured every 6 weeks as cuttings on T medium[22] (see the table) solidified with 0.8% w/v cleaned agar at 26° in 16 hr/day light (1000–2000 lux) in a growth chamber.

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The digest is gently agitated and then left for a further half hour to complete digestion. The solution is filtered through a 100-μm stainless-steel mesh sieve and washed through with one-half volume of 0.6 *M* sucrose (MES, pH 5.6), distributed into capped centrifuge tubes, and centrifuged for 10 min.

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Alternatively the protoplasts can be resuspended in K3A or other media or osmoticum and manipulations such as transformation or fusion carried out (e.g., as described by Paszkowski and Saul[23]).

Culture. The protoplast suspension in H medium is pipetted into petri dishes (1.5 ml/6-cm-diameter petri dish, respectively) and an equal volume of liquified K3A medium (0.3 *M* sucrose, Nagy and Maliga,[2] see the table) containing 1.2% w/v SeaPlaque agarose, cooled to 40°, is added. The protoplasts are distributed evenly by gentle swirling, and the medium allowed to gel.

Petri dishes are sealed with Parafilm or an equivalent and incubated in the dark at 26° overnight, followed by transfer to the light (500 lux) at the same temperature for a further 6 days.

[22] J. P. Nitsch and C. Nitsch, *Science* **163**, 85 (1969).

[23] J. Paszkowski and M. W. Saul, this volume [28].

The agarose gel containing the protoplast-derived cells is cut into quadrants and transferred to 30 ml of liquid medium (1 : 1 mixture of K3A and H460) in 10-cm-diameter containers. These are placed on a gyratory shaker (80 rpm, 1.25 cm throw) in the dark at 26°.[13]

After 1 week one-half of the medium is replaced with a 1 : 1 mixture of K3C and J media (see the table), and thereafter one-half the medium is replaced weekly with a 1 : 1 mixture of K3E and J media. Cultures should be split into 2 containers after 3 weeks and again after 5 weeks where no selection regime is being employed, as otherwise there will be too many colonies per container to allow full development.

Should it be necessary to culture the protoplasts in liquid medium (e.g., after fusion treatments), they can be cultured at 5×10^4–10^5 per ml in K3A medium or a 1 : 1 mixture of this medium and H460 as used above, diluting the culture weekly with the media described or embedding into agarose or agar after 1 week (see Paszkowski and Saul[23]) or at a later time.

Regeneration of Plants. Colonies of 1–2 mm diameter are taken with forceps and placed on Linsmaier and Skoog[24] medium solidified with 0.8% w/v cleaned agar (LS, see the table) and incubated in the dark at 26°.

After 3–5 weeks, depending on the size of the original colony, these should reach 1 cm in diameter. Each colony is then split into 4 parts and 2 placed on fresh LS medium as above, and 2 on LS medium with 0.3 mg/liter BAP as the only phytohormone (regeneration medium). These latter dishes are incubated in the dark for 1 week and thereafter in the light (3000–5000 lux).

Shoots arising from the callus on regeneration medium are cut off when 1–2 cm long and placed on LS medium as above, but without hormones. When the shoots reach 3–5 cm in length they are transferred to T medium and treated as shoot cultures (see above).

They can be transferred to soil once they have an established root system: the agar is gently washed away and the plantlets potted up. They require a humid atmosphere for the first week and can then be hardened off and grown under normal greenhouse conditions.

2. *Preparation and Culture of Leaf Mesophyll Protoplasts from Greenhouse Grown Plants (Petunia hybrida)*

The method described is based on that of Durand *et al.*[25] as described by Shillito *et al.*[13] and further modified by S. Kruger-Lebus[26] in our laboratory for use with the "cyanidin type"[27] and other petunias. A high division frequency and rate of conversion to colonies of protoplasts from a number of *Petunia* species is achieved by this method.

Source of Material. Plants of *Petunia hybrida* and other *Petunia* species are clonally propagated via cuttings, grown in clay pots in a controlled environment [12 hr light (5000 lux), 27/20° day/night, 50/70% relative humidity] and watered morning and evening with commercial fertilizer (0.1% v/v Greenzit).

Preparation of Protoplasts. Young leaves at approximately two-thirds of their final size are washed with tap water and sterilized by immersion for 10 sec in 70% v/v ethanol followed by 10 min in 0.01% w/v $HgCl_2$ solution containing 0.05% w/v Tween 80, and then washed carefully with 5 changes of sterile distilled water (each change 5 min).

Leaf halves without midribs are wet with osmoticum P1 (0.3 *M* mannitol, 0.04 *M* $CaCl_2$, MES, pH 5.8) and arranged in a stack of 6 on the lid of a 9-cm petri dish ready for cutting. They are cut carefully into clean sections 0.5 mm wide, transferred into a small screw top flask containing 10 ml of enzyme solution (2% w/v Cellulase "Onozuka" R10, 1% w/v hemicellulase and 1% w/v pectinol in osmoticum P1), and vacuum infiltrated until the leaf tissue is translucent.

The leaf slices are placed in fresh enzyme solution in a petri dish (0.5 g/10 ml in a 9-cm petri dish), which is sealed with Parafilm and incubated at 28° for ~3 hr. The incubation mixture is checked periodically under the inverted microscope for the release of protoplasts. The time required may vary, especially with greenhouse grown material.

The digest is gently agitated, filtered through a 100-μm mesh stainless-steel sieve, and transferred in 5-ml aliquots into 10–15 ml centrifuge tubes. Osmoticum P2 (0.375 *M* mannitol, 0.05 *M* $CaCl_2$, MES, pH 5.8) is added (5 ml) to each tube and, after gentle mixing, these are centrifuged for 5 min to sediment the protoplasts.

The supernatant is carefully pipetted off, and the sediment is gently shaken to free the protoplasts before resuspension in 10 ml of osmoticum P2. Washing by sedimentation is repeated 2 times. If necessary, the suspension is overlaid on 0.6 *M* sucrose to remove debris and the protoplasts collecting at the interface recovered and resuspended in osmoticum P2. A sample is taken and diluted for counting and the protoplasts sedimented once more and resuspended in medium (K0, see the table) at 10^5 to 2×10^5/ml.

Culture. The suspension is pipetted into 9-cm-diameter petri dishes (3 ml per dish) and 3 ml of liquefied K0 medium containing 1.2% w/v Sea-Plaque agarose at 40° added. The protoplasts are distributed evenly by gentle swirling and the medium allowed to set.

The cultures are incubated for 6 days in the dark at 26°. Half the

24 E. M. Linsmaier and F. Skoog, *Physiol. Plant.* **18**, 100 (1965).
25 J. Durand, I. Potrykus, and G. Donn, *Z. Pflanzenphysiol.* **69**, 26 (1973).
26 S. Kruger-Lebus, unpublished (1984).
27 D. Hess, *Planta* **59**, 567, (1963).

protoplast-containing agarose gel is transferred to each of two 10-cm-diameter containers each containing 40 ml of liquid K0 medium containing a one-tenth concentration of the hormones and with 2% v/v coconut milk. These are incubated at 26° on a gyratory shaker (80 rpm, 0.6 cm throw) in the light (500–1000 lux). The liquid medium is replaced weekly, each time reducing the glucose concentration in the original medium by one-quarter so as to reduce the osmotic pressure.

After 6–8 weeks the colonies are 2–3 mm in size and can be cultured further.

Regeneration of Plants. Colonies are transferred directly to regeneration medium (NT; see the table) with 0.8% w/v SeaPlaque agarose, 2% w/v sucrose, and 1 mg/liter each of NAA and BAP) as they do not grow well as calli.

Where shoot regeneration occurs, this is manifested as a mossy type growth of many shoot primordia. On transfer to the same medium with the addition of 0.1 mg/liter GA3, these normalize, eventually to be rooted, potted on, and moved to the greenhouse.

3. *Preparation and Culture of Protoplasts from Roots of Phaseolus aureus (Mung Bean)*

The use of roots as a source of protoplasts was first described in 1960.[28] However, it is only recently that their use as a source of dividing protoplasts from a number of species has been described. We describe here the protocol of Xhu *et al.*,[29] which this group has recently applied not only to root protoplasts, but also to seedling protoplasts, of a number of grain legumes and Brassica.[30]

Source of Material. Seeds of *Phaseolus aureus* cv. Pism-2 (*Vigna radiata*, mung bean) were surface sterilized with 10% v/v commercial bleach (Domestos; Lever Brothers, Kingston upon Thames, KT12BA, England) for 30 min and washed 3 times with sterile tap water. Sterile seeds were germinated in the dark (27°) for 24–30 hr in 9-cm petri dishes each containing 10 ml sterile tap water (50 seeds per dish).

Preparation of Protoplasts. Root tips (1 cm long) were excised, cut transversely into 0.5–1 mm sections, and plasmolysed for 1 hr in CPW13M solution (see the table). Plasmolysed sections from 100 root pieces were incubated with gentle agitation on a rotatory shaker (16 hr, 25°, 60 rpm) in 4 ml of enzyme solution (2% w/v Rhozyme, 4% w/v Meicelase, 0.03% w/v

[28] E. C. Cocking, *Nature (London)* 187, 927 (1960).
[29] Z.-H. Xhu, M. R. Davey, and E. C. Cocking, *Z. Pflanzenphysiol.* 104, 289 (1981).
[30] M. R. Davey, *in* "Protoplasts 1983: Lecture Proceedings (I. Potrykus, C. T. Harms, A. Hinnen, R. Hutter, P. J. King, and R. D. Shillito, eds.), p. 19. Birkhauser, Basel, 1983.

Macerozyme R10 in CPW13M). Antibiotics were included (400 mg/liter ampicillin, 10 mg/liter gentamycin, 10 mg/liter tetracycline) in the enzyme solution in some cases.

Protoplasts were released by gently squeezing. The enzyme–protoplast mixture was filtered through a nylon sieve (64 μm mesh) and the protoplasts pelleted (10 min). The pellet was resuspended in CPW21S solution (see the table) and centrifuged (5 min). Floating protoplasts were collected and washed twice with CPW13M, resuspended in culture medium, and counted.

Culture. Protoplasts were cultured at $2–2.5 \times 10^4$/ml at 23° in the dark or diffuse light in 56 × 14-mm Nunclon dishes (A/S Nunc, Kamstrup, DK-4000, Roskilde, Denmark) each containing 2 ml of liquid medium or 1 ml of liquid medium over 1 ml of agar (Sigma, 0.5% w/v) solidified medium.

A number of media have been tested. The medium given (modified B5P, see the table) gave good results. B5[31] medium (1 ml) (B5P containing 3% w/v sucrose and no mannitol) was added after 7–10 days. Protoplast-derived colonies were collected after 15–20 days growth and replated in liquid modified White's medium[32] with 0.5 mg/liter each of NAA and Zeatin.

4. *Isolation and Culture of Protoplasts from a Cell Suspension Culture of Daucus carota (Carrot)*

Protoplasts can be isolated and cultured from a wide range of suspension cultures and are a good source of large numbers of uniform protoplasts if regeneration to plants is not necessarily required. We describe a method for use with cultures of carrot (in this case a nonmorphogenic line), which allows one to easily obtain 10^8 protoplasts in one isolation.

Source of Material. Suspension cultures of carrot[33] maintained in a Murashige and Skoog[34] based medium (CS; see the table) by weekly subculture (1 : 7 dilution) and grown on a gyratory shaker (110 rpm, 2 cm throw) at 26° in the dark.

Preparation of Protoplasts. Suspension cultures 2–3 days after transfer are harvested by centrifugation (500 *g*, 5 min), resuspended in two times the original volume of enzyme solution (2% w/v Cellulase "Onozuka" R10, 1% w/v Macerozyme R10, 0.5% w/v Driselase, in 0.4 *M* mannitol, 0.025 *M* $CaCl_2$, MES, pH 5.6) and poured (10 ml/9-cm dish) into

[31] O. L. Gamborg, R. A. Miller, and K. Ohyama, *Exp. Cell Res.* 50, 151 (1968).
[32] H. N. Wood and A. C. Braun, *Proc. Natl. Acad. Sci. U.S.A.* 47, 1907 (1961).
[33] R. H. Smith, this volume [22].
[34] T. Murashige and F. Skoog, *Physiol. Plant.* 15, 473 (1962).

petri dishes. These are sealed and incubated at 24° on a rocking table at 20–30 rpm for 4–5 hr. Preparations are checked regularly for the release of protoplasts.

The digest is filtered through a 50-μm stainless-steel sieve, transferred into capped centrifuge tubes, and sedimented by centrifugation for 5 min. The protoplasts are washed twice by sedimentation with osmoticum (0.29 *M* mannitol, 0.125 *M* $CaCl_2$, MES, pH 5.6), counted, and resuspended in culture medium (CP, see the table) at a population density of 2×10^5/ml.

Culture. The protoplasts can be manipulated in various ways and then cultured in CP medium at this density in liquid medium at 26° in the dark. Cultures are diluted by weekly addition of an equal volume of medium without mannitol to reduce the osmotic pressure.

Alternatively, they can be incorporated into agar or agarose solidified medium and cultured as described for petunia and tobacco protoplasts.

5. *Preparation and Culture of Protoplasts from a Nonmorphogenic Suspension Culture of a Graminaceous Species Lolium multiflorum*

Protoplasts from leaf or other whole plant tissues of grasses do not in general divide in culture although there have been exceptions to this rule.[35] However, there are a number of suspension cultures of graminaceous species available, and these have been used to produce protoplasts which divide. There have been reports of division and colony formation of protoplasts from morphogenic suspension cultures of these species[36] but these have not yet proved to be repeatable.

We describe a protocol developed in our laboratory for the isolation and culture of protoplasts from *Lolium multiflorum* (Italian Ryegrass) suspension culture cells.

Source of Material. The cell line was originally established by P. J. Dale who has also used it for protoplast culture.[37]

Suspension cultures are maintained by weekly serial transfer (1:7 dilution) in CC medium[38] (see the table) without mannitol and with 2 mg/liter 2,4-D on a gyratory shaker (110 rpm, 2 cm throw) in low light levels (500 lux).

Preparation of Protoplasts. Cultures are used for protoplasting 4, 5, or 6 days after subculture. Ten milliliters of cells is sedimented by centrifugation (5 min) and resuspended in the same volume of enzyme solution (4% w/v Driselase in 0.38 *M* mannitol, 8 m*M* $CaCl_2$, MES, pH 5.6).

35 I. Potrykus, *in* "Advances in Protoplast Research" (L. Ferenczy and G. L. Farkas, eds.), p. 243. Hung. Acad. Sci., Budapest, 1980.

36 V. Vasil and I. K. Vasil, *Theor. Appl. Genet.* **56**, 97 (1980).

37 M. G. K. Jones and P. J. Dale, *Z. Pflanzenphysiol.* **105**, 267 (1982).

38 I. Potrykus, C. T. Harms, and H. Loerz, *Theor. Appl. Genet.* **54**, 209 (1979).

The solution is poured into a 9-cm petri dish and this is sealed and placed on a rocking table for 1 hr at 20° before being incubated overnight (15 hr) without agitation at the same temperature. The preparation is then placed on the rocking table for an hour followed by another hour without agitation.

The protoplasts are filtered through a 100-μm mesh stainless-steel sieve, an equal volume of 0.2 *M* $CaCl_2$ (MES, pH 5.8) added, and the suspension distributed into 2 centrifuge tubes. After centrifugation to sediment the protoplasts (10 min) they are taken up in 3 ml osmoticum L1 (0.25 *M* mannitol, 0.1 *M* $CaCl_2$, MES, pH 5.8) and overlayered on a 5 ml sucrose cushion (0.6 *M* sucrose, MES, pH 5.8).

Protoplasts collecting at the interface after centrifugation are carefully removed and washed twice with osmoticum L1, counted, and resuspended in CC medium (see the table) at a density of 2×10^6/ml.

Culture. The protoplasts are cultured in 3.5-cm-diameter petri dishes (2 ml per dish) at 26° in the dark. Fresh CC medium with 0.2 *M* mannitol is added after 7 days to dilute the culture and reduce the osmotic pressure, and medium without mannitol added weekly thereafter.

Calli arising from the cultures are grown on CC medium as used for the suspension cultures solidified with 0.8% w/v cleaned agar.

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Table of Contents

[22] Establishment of Calli and Suspension Cultures[1]

By Roberta H. Smith

A plant callus is a wound response from an explant (the fragment of a plant or tissue used to initiate a culture) consisting of unorganized, dividing cells. Cellular proliferation can also be produced *in vitro* without physical injury or wounding by germinating seeds on a medium containing a plant growth regulator. Individual cells in a callus mass can vary in size, shape, pigmentation, and appearance. Most are differentiated cells with a large central vacuole and nucleus to one side as opposed to undifferentiated, meristematic cells which are isodiametric, small, lack a prominent vacuole, are cytoplasmic, and have a large central nucleus.

Callus cultures were first reported in 1939 independently by three laboratories. Gautheret[1a] and Nobécourt[2] used carrot (*Daucus carota* L.) root tissues with 3-indoleacetic acid (IAA) to obtain callus growth. White[3] used procambial tissue from a tobacco hybrid (*Nicotiana glauca* × *N. langsdorffii*) to establish callus. Since then, callus cultures have been used for studies of cell division, morphogenesis, secondary product synthesis, bioassays for plant growth regulators, and more recently for selection, mutation, and genetic modification at the cellular level.

As callus cultures are subcultured (divided and placed on fresh medium, usually at 4-week intervals) many things can happen to change the original callus composition. Some of the cell types within a culture will divide faster than others and tend to overgrow and become the predominate cellular type composing a culture. Friability may change as a result. The ploidy of a culture may change with time.[4] The cells may lose their requirement for plant growth regulators (i.e., become habituated). Secondary metabolite production can change. If plants are regenerated from successive subcultures, the incidence of variant plants (somaclonal variation)[5] can increase.

Callus and suspension culture initiation involves three major considerations—selection of explant, medium, and culture conditions—each one

[1] Texas Agricultural Experiment Station manuscript No. 19909.
[1a] R. J. Gautheret, *C.R. Hebd. Seances Acad. Sci.* **208**, 118 (1939).
[2] P. Nobécourt, *C.R. Seances Soc. Biol.* **130**, 1270 (1939).
[3] P. R. White, *Am. J. Bot.* **26**, 59 (1939).
[4] T. Murashige and R. Nakano, *Am. J. Bot.* **54**, 9763 (1967).
[5] P. J. Larkin and W. R. Scowcroft, *Theor. Appl. Genet.* **60**, 197 (1981).

of which is important to the success of the attempt.[6] The explant used to initiate the culture can be derived from any piece of the vegetative or flowering plant. Certain explants, however, are easier to obtain year round, are easier to establish as aseptic cultures, and respond faster for callus induction.[7] Culture media vary as there is no single formulation which will universally work for all plant species.[8] The culture environment can be modified in photoperiod, light quality and intensity, and temperature. Some general techniques will be described which have worked for various plant tissues; however, successful induction of callus from other sources may involve empirical manipulation of explant, culture medium, and culture environment.

Explant Procedure

Seedling tissue arising from the aseptically germinated seed is a choice source of explant tissue for carrot and tobacco callus induction. The method of surface sterilization which follows can be applied for surface sterilization of most explant tissue. It is more difficult to obtain clean cultures from some explants than others. The concentration of bleach and length of exposure can be varied. For a discussion of isolation of other explant sources and sterilizing agents, refer to Street,[6] Yeoman,[9] and Murashige.[10]

Laboratory Protocol: Explant Preparation: Aseptic Germination of Seeds

Seed Germination Medium

1. 25 × 150 mm culture tubes or petri dishes containing 25 ml of germination medium (see Medium Preparation section to prepare stocks), the composition of which is as follows: 10 ml each of the inorganic salt stocks, 30 g sucrose, dilute to 1 liter, adjust pH to 5.7, add 8 g of agar, and heat until agar dissolves; pour 25 ml each into tubes or petri dishes.
2. Rinse water (200 ml) in a 500-ml Erlenmeyer flask, capped with aluminum foil.
3. Petri dishes in a cannister.

[6] H. E. Street, *in* "Plant Tissue and Cell Culture" (H. E. Street, ed.), p. 1. Univ. of California Press, Berkeley, 1973.
[7] T. Murashige, *TCA Rep.* 12(2), 41 (1978).
[8] Murashige, *HortScience* 12(2), 3 (1977).
[9] M. M. Yeoman, *in* "Plant Tissue and Cell Culture" (H. E. Street, ed.), p. 31. Univ. of California Press, Berkeley, 1973.
[10] T. Murashige, *Annu. Rev. Plant Physiol.* 25, 135 (1974).

4. Autoclave seed germination medium for 15 min and rinse water and petri dishes for 20 min at 121°.

Sterilization of Seeds

Carrot

1. Wrap the seeds in several layers of ca. 10 × 10-cm squares of cheesecloth.
2. Place the wrapped seeds in a 25 × 150-mm culture tube, add 70% ethanol, shake or swirl for ~2 min, and then decant the alcohol.
3. Disinfect the wrapped seeds in a 20% (v/v) solution of commercial bleach or in a solution composed of 1.05% sodium hypochlorite and 2 drops of Tween-20 per 100 ml total volume. Pour the disinfectant into the cap of the test tube and then into the culture tube, completely covering the cheesecloth-wrapped seeds. Cap the tube.
4. Swirl the tube intermittently on a vortex mixer for 20 min. Using aseptic procedures, place the materials in a laminar air flow hood, pour off the disinfectant, and rinse the seeds 3 times with sterile water.

Note: Sometimes carrot seeds are very contaminated: therefore, it is prudent to plant only 2–3 seeds per dish or tube. Seed contamination will be apparent on the seed germination medium after about 5 days.

Tobacco

1. Put the seeds in a 15-ml screw-cap, conical centrifuge tube and add 0.75% sodium hypochlorite (containing 2 drops Tween-20/100 ml).
2. Swirl the tube intermittently on a vortex mixer for 20 min.
3. Allow the seeds to settle or centrifuge at low speed.
4. Pour off the disinfecting solution and rinse 3 times with sterile water.

Seed Germination

1. Using long-handled forceps (Pott Smith dressing forceps), plant 1–3 seeds per culture tube on the surface of the agar. To sterilize forceps, immerse in 70% alcohol 10 min. Then place tips in 95% alcohol covering ca. one-half the length of the forceps. Prior to using, remove from alcohol and pass forceps tips through a flame. Allow alcohol to burn off and forceps to cool prior to touching plant tissue. (Caution: hold forceps close to horizontal so flame does not burn hand.) Note: Many beginners will kill the plant tissue by not allowing the instruments to cool.
2. Place cultures in 16 : 8 hr (light : dark) photoperiod at light intensity of 50–100 $\mu Em^{-2} sec^{-1}$ and temperature 26 ± 3°.

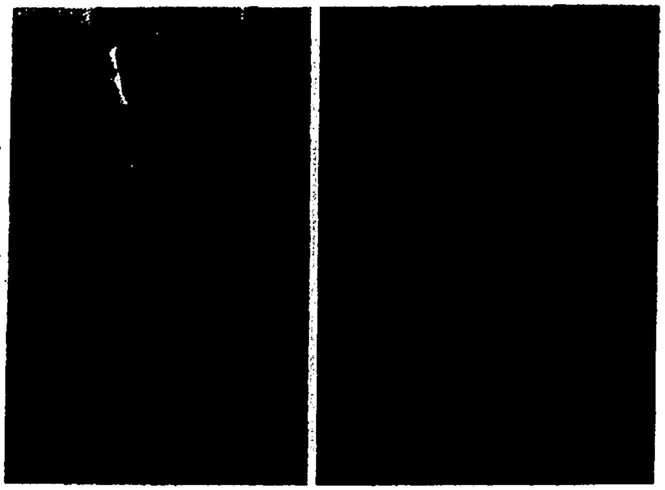

FIG. 1. Seedlings of carrot (left) and tobacco (right) aseptically germinated.

3. In 1–3 weeks seedlings from aseptically germinated seeds will be ready for callus induction; all parts of the seedlings can be used as an explant for callus induction (Fig. 1).

Nutrient Medium

Components of a nutrient medium for callus growth generally include inorganic salts, a carbohydrate, vitamins, plant growth regulators, and organic supplements (sometimes optional). Inorganic salts, vitamins, and plant growth regulator stock solutions are prepared in concentrated form, and usually several inorganic salts and vitamins can be combined to minimize the number of stocks. Plant cell culture medium is a rich substrate which will support growth of bacteria and fungi. Generally, addition of fungicides and bactericides to medium can result in complications and phytotoxicity to the explant.[11] Stock solutions enhance accuracy and efficiency of medium preparation. Storage of salt stocks is best under refrigeration. Always use glass-distilled or demineralized water to prepare stocks and media. Detailed discussions of common media are proved elsewhere.[12–16]

[11] K. C. Thurston, S. J. Spencer, and U. Arditti, *Am. J. Bot.* **66**, 825 (1979).
[12] O. L. Gamborg, R. A. Miller, and K. Ojima, *Exp. Cell Res.* **50**, 151 (1968).
[13] O. L. Gamborg, T. Murashige, T. A. Thorpe, and I. K. Vasil, *In Vitro* **12**, 473 (1976).

Laboratory Protocol: Medium Preparation

Stock Solutions

1. Inorganic salts of Murashige and Skoog,[15] 100 times final medium concentration (grams/liter stock):
   - Nitrates
     - NH_4NO_3, 165
     - KNO_3, 190
   - Sulfates
     - $MgSO_4 \cdot 7H_2O$, 37
     - $MnSO_4 \cdot H_2O$, 1.690
     - $ZnSO_4 \cdot 7H_2O$, 0.860
     - $CuSO_4 \cdot 5H_2O$, 0.0025
   - Halides
     - $CaCl_2 \cdot 2H_2O$, 44
     - KI, 0.083
     - $CoCl_2 \cdot 6H_2O$, 0.0025
   - PO_4, BO_3, MoO_4
     - KH_2PO_4, 17
     - H_3BO_3, 0.620
     - $NaMoO_4 \cdot 2H_2O$, 0.025
   - NaFe EDTA
     - $FeSO_4 \cdot 7H_2O$, 2.784
     - Na_2EDTA, 3.724
     - Store in amber bottle

2. Plant growth regulators (store in refrigerator): (1) 2,4-dichlorophenoxyacetic acid (2,4-D) and IAA stocks. Weigh out 10 mg auxin into a 200 ml beaker, add several drops of 1 *N* NaOH or KOH until crystals are dissolved (not more than 0.3 ml), rapidly add 90 ml of double distilled H_2O, bring up to 100 ml volume in a volumetric flask. Make IAA stock fresh weekly. (2) Kinetin stock: same as for auxins except use 1 *N* HCl and a few drops of H_2O with gentle heating to dissolve crystals.

3. Vitamins (store in freezer in 10 ml aliquots) (mg/100 ml stock):
   - Nicotinic acid, 5
   - Thiamin–HCl, 4
   - Pyridoxine–HCl, 5

[14] L. C. Huang and T. Murashige, *Tissue Cult. Assoc. Man.* **3**, 539 (1977).
[15] T. Murashige and F. Skoog, *Physiol. Plant.* **15**, 473 (1962).
[16] T. Murashige, *in* "Tissue Culture Methods and Applications" (P. F. Kruse and M. K. Patterson, Jr., eds.), p. 698. Academic Press, New York, 1973.

Medium, 1 Liter

1. In a 2-liter Erlenmeyer flask add 500 ml of deionized, distilled water and mix in the following:

Carrot[17–19]

10 ml each of the five inorganic salt stocks
30 g sucrose
10 ml vitamin stock
100 mg myo-inositol
1 ml 2,4-D stock, (0.5 μM)

Tobacco[6,15,18–20]

10 ml each of the inorganic salt stocks
30 g sucrose
10 ml vitamins
100 mg myo-inositol
2 ml kinetin stock (0.9 μM)
20 ml IAA stock (11.4 μM)
(Leaf or stem explants of *Nicotiana* spp. have been used to initiate callus on MS medium with 4.5 μM 2,4-D and 2 g/l casein hydrolysate.)[18]

2. Final pH adjusted to 5.7 with 1 *N* KOH, NaOH, or HCl. Add 8 g of agar.

Heat and stir while melting the agar so that the agar does not burn on the bottom of the flask. Distribute 25 ml/culture tube. An alternate procedure to melt the agar is to autoclave the flask 3–7 min at 121°.[14] After melting and autoclaving (15 min at 121°C), distribute 25 ml per sterile, plastic petri dish in a laminar air flow hood.

Care should be taken in autoclaving media. Sugars will undergo caramelization if autoclaved too long, and will also react with amino compounds (Maillard Reaction).[21] These then form brown, high-molecular-weight materials which can inhibit growth. A brown color to the medium

[17] D. F. Wetherell, *in* "Propagation of Higher Plants through Tissue Culture" (K. W. Hughes, R. Henke, and M. Constantin, eds.), p. 102. Tech. Inf. Cent., USDE, 1978.
[18] D. A. Evans, W. R. Sharp, and C. E. Flick, *in* "Horticultural Reviews" (J. Janik, ed.), p. 214. AVI Publ. Co., Westport, Connecticut, 1980.
[19] O. L. Gamborg and L. R. Wetter, *in* "Plant Tissue Culture Methods" (O. L. Gamborg and L. R. Wetter, eds.), p. 95. Natl. Res. Counc. Can., 1975.
[20] J. P. Helgeson, *in* "Nicotiana: Procedures for Experimental Use" (R. D. Durbin, ed.), U.S. Dept. Agric., Tech. Bull. No. 1586, p. 52. USDA, Washington, D.C., 1979.
[21] H. G. Peer, *in* "Effects of Sterilization on Components in Nutrient Media" (J. van Bragt, D. A. A. Mossel, P. L. M. Pierik, and H. Veldstra, eds.), Misc. Pap. No. 9, p. 105.

FIG. 2. Carrot explants ready to be placed in culture.

is apparent if media is autoclaved too long or left in the autoclave to cool down. Media autoclaving should be accurately timed, and removed from the autoclave promptly when the temperature is just below 100° and pressure is zero.

Media can be stored 1–2 weeks prior to use, preferably under refrigeration in the dark. Media with IAA cannot be stored as IAA breaks down.

Laboratory Protocol: Culture of Primary Explants for Callus Induction

1. Carrot explants approximately 1 cm long from seedling roots and petioles are excised and placed on the medium (Fig. 2).
2. Tobacco explants from cross-sections of leaf and stem tissue are excised and placed on the medium.
3. All cultures are incubated in the dark at 27°.
4. After 4–6 weeks callus can be separated from the original explant and subcultured onto fresh medium for further growth (Fig. 3). The stock callus cultures should be subdivided and cultured at 4- to 6-week intervals (Fig. 4).

Sometimes after 3–5 days in culture, bacterial and/or fungal growth will be apparent. Contamination that results from the explant (spores on

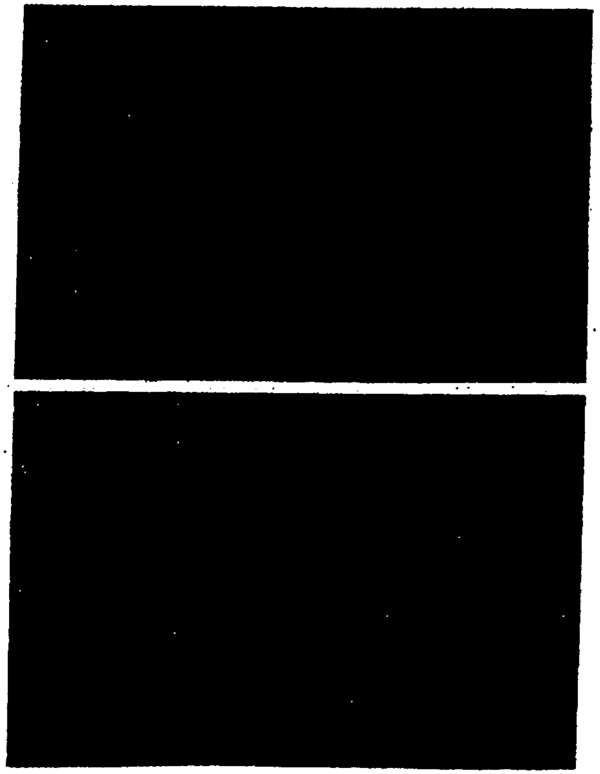

FIG. 3. Carrot (top) and tobacco (bottom) callus beginning to form from the explants after about a week in culture.

tissue, rinse water, petri dish, instruments) will arise on the medium adjacent to the explant. Contamination scattered on the agar surface usually results from improperly sterilized media, contaminated air flow hood, or poor sterile technique.

For details on media for callus induction for various other dicots and monocots (see Evans *et al.*[18]).

Suspension Culture

Suspension cultures (cell cultures dispersed in moving liquid medium)[6] are established from friable callus cultures. Friable callus can be visually selected and subcultured from compact, hard cultures or can be achieved

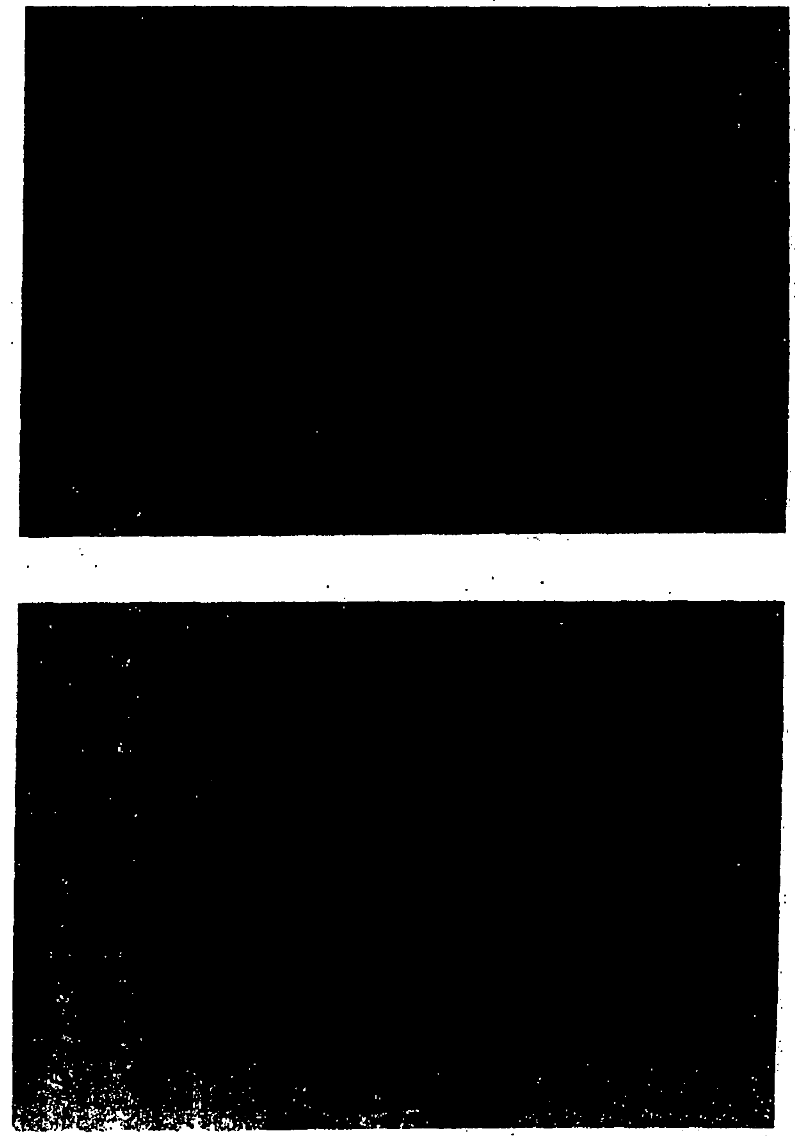

FIG. 4. Carrot (top) and tobacco (bottom) stock callus cultures.

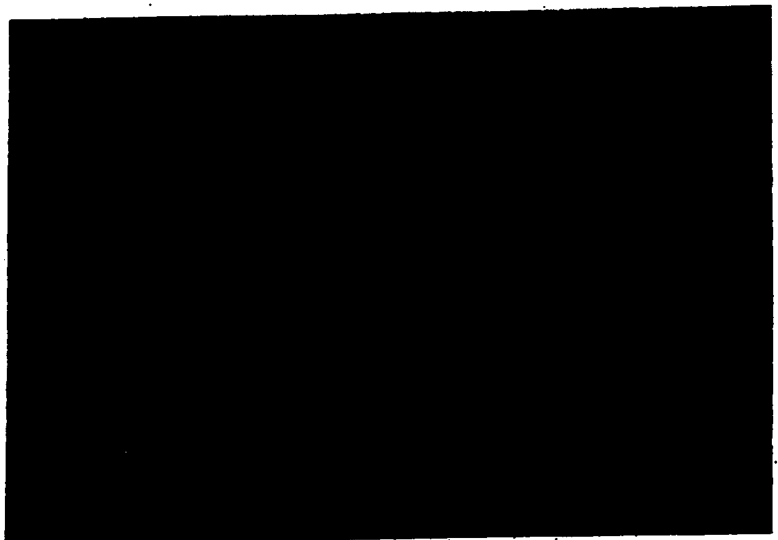

FIG. 5. Carrot suspension culture after about 10 days growth.

by manipulating medium components including the type or concentration of auxin,[22] cytokinin, or casein hydrolysate.[8] Environmental culture conditions may also influence friability.

Suspension cultures of carrot (Fig. 5) can be initiated by subculturing the carrot callus onto liquid medium of the same composition as callus medium except lacking agar. Baffled, long-necked Ehrlenmeyer flasks are commonly used and placed on an orbital or gyratory shaker at 50–120 rpm. Tobacco suspension cultures can be cultured on the same callus induction basal medium, substituting 1 μM 2,4-D for IAA and deleting agar.

Suspension cultures are initiated by an inoculum which establishes a cell density of $0.5–2.5 \times 10^5$ cells per ml.[23] If too low a density inoculum is used, the culture may not grow. Generally, 2–3 g of friable callus is used, the culture may not grow. Generally, 2–3 g of friable callus is inoculated onto 100 ml of liquid medium. Small clumps of cells will result after 2 to 3 weeks. Suspension cultures require more frequent subcultures at 1- to 3-week intervals, depending on the amount of callus used to

[22] J. G. Torrey and J. Reinert, *Plant Physiol.* 36, 483 (1961).

[23] H. E. Street, *in* "Plant Tissue and Cell Culture" (H. E. Street, ed.), p. 59. Univ. of California Press, Berkeley, 1973.

initiate the culture and the vigor of the culture. A pipette with a wide tip can be used to inoculate new cultures or the old suspension culture can be swirled and a specified volume rapidly poured into a sterile, graduate cylinder. The contents of the cylinder is then poured into the new medium. According to Helgeson,[20] if cell concentrations exceed 40 mg fresh weight per milliliter of medium, transfers with no appreciable lag before the logarithmic phase can be established. A fine dispersion of single cells and small cell clusters can be established and maintained by screening the suspension culture through a nylon or wire mesh to exclude larger cell clumps. Agar medium cultures can be initiated from suspension cultures by pipetting 1–3 ml of suspension culture onto the surface of an agar-solidified petri dish.

Growth Curve

For either a suspension or callus culture it is important to establish a growth curve. Growth curves can be established by inoculating cultures with a specified volume or weight of cells and then taking daily interval measurements. Fresh weight, dry weight, and mitotic index[9,19,23] can be used to follow culture growth. Suspension-culture-packed cell volume (volume of cells per unit volume of culture) measurement is also a rapid method to follow growth.[22] A specified aliquot of suspension culture is centrifuged in a 15-ml graduated conical centrifuge tube at 2000 *g* for 5 min. A measurement in milliliters of the packed cell volume per milliliter culture can be followed. A growth curve with cell number, weight, etc., on the *y* axis and time intervals on the *x* axis will have 5 segments: the lag, exponential, linear, progressive decelerating, and stationary.[23] Generally, a culture should be subcultured prior to reaching stationary phase.